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Immunology

Down-regulation of NF-κB protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D_3

(transcription factors/vitamin D_3/rel proteins)

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ABSTRACT **The effect of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], a steroid hormone with immunomodulating properties, on nuclear factor κB (NF-κB) proteins was examined in *in vitro* activated normal human lymphocytes by Western blot analysis. Over a 72-hr period of activation, the expression of the 50-kDa NF-κB, p50, and its precursor, p105, was increased progressively. When cells were activated in the presence of 1,25$(OH)_2D_3$, the levels of the mature protein as well as its precursor were decreased. The effect of the hormone on the levels of p50 was demonstrable in the cytosolic and nuclear compartments; it required between 4 and 8 hr and was specific, as 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 were ineffective. Besides p50, 1,25$(OH)_2D_3$ decreased the levels of another NF-κB protein, namely c-rel. In addition, 1,25$(OH)_2D_3$ decreased the abundance of a specific DNA–protein complex formed upon incubation of nuclear extracts from activated lymphocytes with a labeled NF-κB DNA binding motif. Further, 1,25$(OH)_2D_3$ inhibited the transcriptional activity of NF-κB in Jurkat cells transiently transfected with a construct containing four tandem repeats of the NF-κB binding sequence of the immunoglobulin κ light chain gene linked to the chloramphenicol acetyltransferase reporter gene. These observations demonstrate directly that there is *de novo* synthesis of NF-κB during human lymphocyte activation and suggest that this process is hormonally regulated.**

Besides its role in calcium and skeletal homeostasis, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] plays a widespread role in the differentiation, proliferation, biosynthetic activity, and function of cells of the immune system (1). Its effects on lymphocytes are mediated by the specific 1,25$(OH)_2D_3$ receptor, a ligand-dependent transcription factor that recognizes DNA elements in the promoters of its target genes, termed vitamin D response elements (VDREs). Nonetheless, VDREs are not present in the regulatory sequences of genes influenced by 1,25$(OH)_2D_3$ in lymphocytes. Further, the effects of 1,25$(OH)_2D_3$ on lymphocytes are pleiotropic and depend on the mode of activation (1). Based on these considerations, we have hypothesized that 1,25$(OH)_2D_3$ exerts its actions on lymphocyte products through interactions with other factors generated following activation, rather than through direct interaction of its receptor with these genes. A precedent for modulation of gene expression by a steroid hormone through influencing other transcription factors has been set in the case of the glucocorticoid receptor (2, 3).

Nuclear factor κ B (NF-κB) designates a family of structurally and functionally related protein complexes used by lymphoid cells to regulate genes encoding products that participate in the immune response (4). All members of the family share a 300-amino acid region of homology that mediates dimerization and high-affinity binding to decameric DNA sequences of the consensus 5′-GGGRNNYYCC-3′ (where R is purine and Y is pyrimidine) (5). The ability of NF-κB to bind DNA is controlled by protein–protein interactions with highly specific inhibitor proteins, termed IκB (6). Association with IκB prevents nuclear uptake by retaining NF-κB complexes in the cytoplasm. Stimulation with various agents causes the release of IκB from the rel–NF-κB complexes, thus allowing the rapid translocation of rel-NF-κB into the nucleus.

The prototypical NF-κB is composed of two distinct subunits of 50 kDa and 65 kDa referred to as p50 and p65 or RelA, respectively. The p50 subunit is synthesized as a 105-kDa protein, p105, that undergoes posttranslational processing to the mature form (7, 8). c-rel, the normal cellular homolog of the oncogene product of the avian reticuloendotheliosis virus REV-T v-rel, is another member of the NF-κB family that is expressed in mammalian cells (9). p50, c-rel, and p65 are in fact the major components of NF-κB complexes, binding to most of the identified cis-acting κB sites. All these proteins are expressed in various cells, but most abundantly in differentiated human lymphoid cell lines and human peripheral blood resting B and activated T cells (4, 10). Though all of them are constitutively expressed, their expression is up-regulated by NF-κB-activated conditions such as those occurring during stimulation of T cells or B cells (11).

Control of the activation of preexisting forms of NF-κB has attracted large attention. However, relatively little is known about the regulation of the level of expression of the various NF-κB proteins themselves. In this report, we present evidence indicating that during normal T-cell activation *in vitro*, there is a progressive increase in the levels of p50, its p105 precursor, and c-rel and that 1,25$(OH)_2D_3$ acts to down-regulate the levels of these proteins and thereby decrease their transcriptional activity in human lymphocytes.

EXPERIMENTAL PROCEDURES

Materials. ^{125}I-labeled protein A (30 mCi/mg; 1 Ci = 37 GBq), [γ-^{32}P]ATP (3000 Ci/mmol), and D-[dichloroacetyl-1-^{14}C]chloramphenicol (50–60 mCi/mmol) were purchased from Amersham. 1,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$], and 25-hydroxyvitamin D_3 [25$(OH)D_3$] were gifts from Milan Uskokovic (Hoffmann–LaRoche). Phytohemagglutinin (PHA) and triamcinolone acetonide (TRM) were purchased from Sigma. The OKT3 monoclonal antibody (Ab) to the T-cell antigen receptor was generated from the ATCC-CRL 8001 hybridoma cells. Antisera to rel-related proteins developed in rabbits were provided by Nancy Rice (National Cancer Institute, Frederick, MD) (12, 13). Anti-SP1157 rec-

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Abbreviations: NF-κB, nuclear factor κB; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25$(OH)D_3$, 25-hydroxyvitamin D_3; TRM, triamcinolone acetonide; PBMC, peripheral blood mononuclear cell; PMSF, phenylmethylsulfonyl fluoride; IL, interleukin; PHA, phytohemagglutinin; CAT, chloramphenicol acetyltransferase; Ab, antibody.

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ognizes the p50 subunit of NF-κB and it also reacts with the precursor of this protein, p105; anti-SP265 recognizes the extreme C terminus of the 75-kDa c-rel protein. The chloramphenicol acetyltransferase (CAT) reporter plasmid, p(NF-κB)$_4$-CAT, was constructed by inserting four tandem copies of the NF-κB binding sequence (5′-gatcCAGAGGGGACTTTCCGAGA-3′) of the immunoglobulin κ light chain gene at a *Bam*HI site upstream of the TATA box of pE1BTATA-CAT (14) and was provided by Michael Su (Vertex Pharmaceuticals, Cambridge, MA).

Cells and Culture Conditions. Peripheral blood mononuclear cells (PBMCs) from normal volunteers or buffy coats were isolated by Ficoll/Hypaque (15) and stimulated with PHA (2 μg/ml), with OKT3 (1:250 dilution), or with other reagents as indicated. The human T-cell leukemia cell line Jurkat was maintained in RPMI 1640 medium in the presence of 10% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Preparation of Subcellular Fractions. At the end of the incubation, cells were washed with phosphate-buffered saline (PBS), resuspended at 4°C in 10 mM Tris·HCl, pH 7.4/1.5 mM EDTA/5 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride (PMSF)/0.3 M KCl, and sonicated. Cytosol fractions were obtained by centrifugation for 1 hr at 105,000 × *g* and precipitated with ammonium sulfate (40% of saturation). Pellets were collected and solubilized in 10 mM Tris·HCl, pH 7.4/1.5 mM EDTA/5 mM dithiothreitol/1 mM PMSF. For the experiments in which we used nuclear extracts (Fig. 5) or nuclear and cytosolic extracts (Fig. 1), isolation of nuclear fractions was performed as described (16). All buffers contained 0.5 mM PMSF, 1 mg of leupeptin per ml, 0.5 mg of pepstatin per ml, and 1 mg of aprotinin per ml. Briefly, 4–6 × 10^7 cells were washed with hypotonic buffer (10 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol) and lysed for 10 min on ice in hypotonic buffer containing 0.1% Nonidet P-40 (30 μl per 10^7 cells). Lysates were centrifuged (10,000 × *g*) at 4°C for 10 min. Supernatants were collected and centrifuged at 100,000 × *g* at 4°C, and the resulting high-speed supernatant was used as the cytosolic fraction. Pelleted nuclei were resuspended in 100 μl of lysis buffer (20 mM Hepes, pH 7.9/420 mM NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/25% glycerol) and incubated at 4°C for 15 min. Lysed nuclei were dispersed in a Vortex and centrifuged at 10,000 × *g*, 4°C for 10 min; supernatants were collected, snap-frozen, and stored at −70°C. Protein concentrations of nuclear and cytosolic fractions were determined by the method of Bradford (17).

Western Blot Analysis. Protein extracts (100 μg per lane) were electrophoresed on SDS/polyacrylamide (7.5%) gels (18), transferred to nitrocellulose membranes, and immunoblotted with the indicated antibodies as described (15). Radioactivity of the bands was determined using a β scanner (AMBIS Systems). Background was subtracted from each lane.

Electrophoretic Mobility Shift Assay. Nuclear extracts from lymphocytes were prepared as described above. A synthetic double-stranded oligonucleotide containing the NF-κB binding sequence of the human interleukin 6 (IL-6) promoter (−82 bp to −47 bp, 5′-ATCAAATGTGGGATTTTCCCATGAGTCTCAATATTA-3′) was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. One nanogram of end-labeled probe was incubated for 20 min at room temperature with 5 μg of nuclear protein in a solution containing 50 μg of poly(dI-dC) per ml, 50 μg of double-stranded salmon sperm DNA per ml, 6% glycerol, 10 mM Hepes (pH 7.5), 80 mM KCl, 1 mM EDTA, and 1 mM EGTA. Competition studies were performed by incubating nuclear extracts with unlabeled NF-κB oligonucleotide or a 22-mer oligonucleotide containing an AP-1 binding motif (5′-CTAGTGATGAGTCAGCCGGATC-3′) for 15 min at room temperature (as indicated in Fig. 5), followed by a 30-min incubation in the presence of the probe. Samples were analyzed on native 5% polyacrylamide gels.

Transfection and CAT Assay. Jurkat cells (1 × 10^7) were transfected in 2 ml of RPMI 1640 medium containing 0.05 M Tris·HCl (pH 7.5), 0.25 mg of DEAE-dextran per ml (Pharmacia), and 2 μg of either the p(NF-κB)$_4$-CAT plasmid or a plasmid containing the CAT gene driven by the Rous sarcoma virus promoter (pRSV-CAT) as a positive control. After incubation at room temperature for 20 min, transfected cells were diluted with RPMI 1640 medium, pelleted, and resuspended in the same medium containing 10% FBS. Cells were maintained in the absence or presence of 10^{-8} M 1,25(OH)$_2$D$_3$ for 48 hr, stimulated with PHA (2 μg/ml) for the last 8 hr of the 48-hr period, and harvested by centrifugation, and lysates were prepared by three cycles of freeze/thaw in 0.1 M Tris·HCl (pH 7.5). CAT enzyme activity was assayed as described (19), and values are expressed as percentage of acetylated chloramphenicol.

RESULTS

Analysis of the expression of NF-κB, prior to and during activation of normal human PBMCs with PHA over a period of 72 hr, was performed using an Ab (SP1157) recognizing the p50 subunit of NF-κB as well as the p105 precursor of this protein (13) (Fig. 1). In either the cytosolic or the nuclear fraction, p50 or p105 was undetectable prior to or following 12 hr of activation. However, following 24 hr of activation, the p50 was detected in both fractions. Thereafter, its expression increased progressively. Activation also led to the appearance of the p105 precursor of p50 in the cytosol and the levels of this protein increased progressively during the activation period. In this particular experiment, the intensity of the p105 signal appeared weaker than that of the p50; however, this was not a consistent finding in subsequent experiments. Addition of 10^{-8} M 1,25(OH)$_2$D$_3$ simultaneously with addition of PHA caused a significant decrease in the expression of p50 at all time points examined. A similar effect was observed in the cytosolic and the nuclear fractions. In addition, 1,25(OH)$_2$D$_3$ decreased the expression of the p105 precursor of p50 in the cytosolic fraction.

Vitamin D metabolites with lesser potency [and lower affinity for the 1,25(OH)$_2$D$_3$ receptor] had no appreciable effects on the level of expression of NF-κB following a 72-hr activation with PHA (Fig. 2). Nonetheless, the synthetic glucocorticoid TRM also inhibited NF-κB expression. In this experiment and that shown in Fig. 3, as opposed to the data shown in Fig. 1, a small amount of p50 could be detected in resting cells and the p105 signal was at least as intense as that

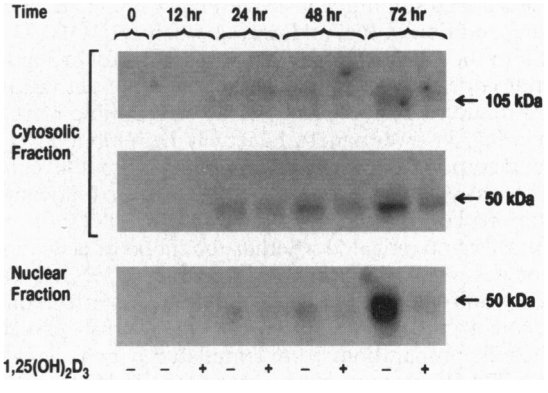

FIG. 1. Effects of 1,25(OH)$_2$D$_3$ on NF-κB expression in PHA-activated human lymphocytes. PBMCs were incubated with PHA alone or in the presence of 10^{-8} M 1,25(OH)$_2$D$_3$ and collected at different times after activation; subcellular fractions were then prepared. Western blotting was performed using the antibody SP1157.

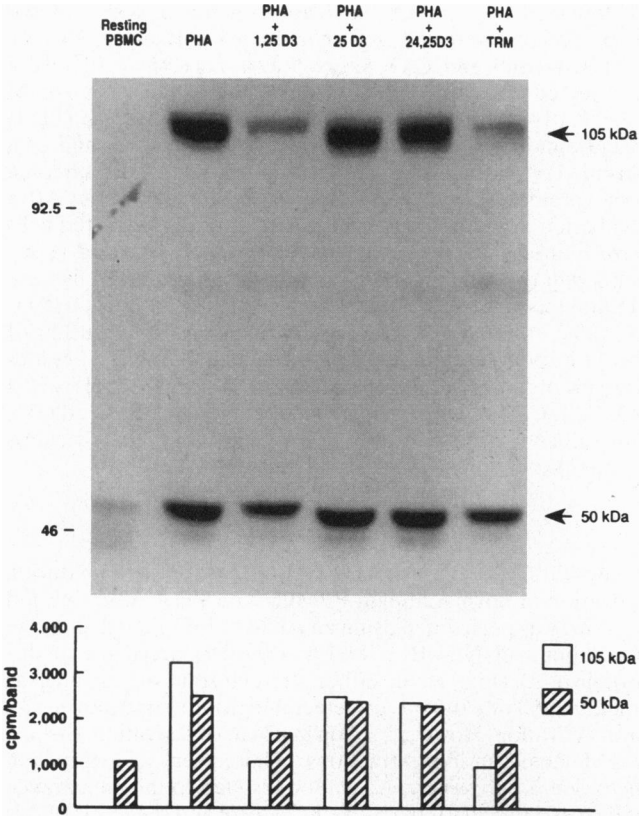

FIG. 2. Steroid specificity of inhibition of NF-κB in PHA-activated human lymphocytes. PBMCs were incubated for 72 hr without stimuli or with PHA alone or in the presence of 10^{-8} M $1,25(OH)_2D_3$, $25(OH)D_3$, $24,25(OH)_2D_3$, or TRM. Western blotting of cytosolic proteins was performed using the SP1157 antibody. Bars represent radioactivity per band. Positions of molecular mass standards are indicated at the left.

of p50. Since in each experiment we have utilized PBMCs from a different donor, we think that this apparent difference is probably due to a difference either in the number of already activated (*in vivo*) lymphocytes or in the levels of constitutive expression of these proteins among various subjects.

To determine the time required for $1,25(OH)_2D_3$ to exert its inhibitory effect on p50 and p105, PBMCs were activated for 72 hr with PHA, and $1,25(OH)_2D_3$ was added either simultaneously with addition of PHA or 24, 48, 64, 68, or 70 hr following addition of PHA (Fig. 3). The inhibiting effect of $1,25(OH)_2D_3$ on p50 and p105 was apparent when the hormone was added simultaneously with PHA or 24, 48, or 64 hr following addition of PHA. However, when $1,25(OH)_2D_3$ was added into the culture for the last 4 hr (68 hr following PHA activation) or the last 2 hr (70 hr following PHA activation) of the 72-hr culture period, there was no discernible effect. As shown in Fig. 3 (lower panel), $1,25(OH)_2D_3$ inhibited [^{3}H]thymidine incorporation when it was added into the cultures simultaneously with addition of PHA or 24 hr following the addition of PHA.

Further, we investigated whether the mode of activation of lymphocytes would influence the effects of $1,25(OH)_2D_3$ on p50/p105 and whether $1,25(OH)_2D_3$ had an effect on the expression of a related protein with NF-κB activity—c-rel (Fig. 4). Cytosolic preparations from stimulated lymphocytes contained a 75-kDa protein that reacted with an Ab recognizing the extreme C terminus of c-rel (Ab SP265) as well as the p105 and p50, in PHA- and OKT3-activated cells. The levels of all three proteins were decreased in the presence of $1,25(OH)_2D_3$. Identical results were obtained using an alternative Ab that recognizes the N terminus portion of c-rel (not shown).

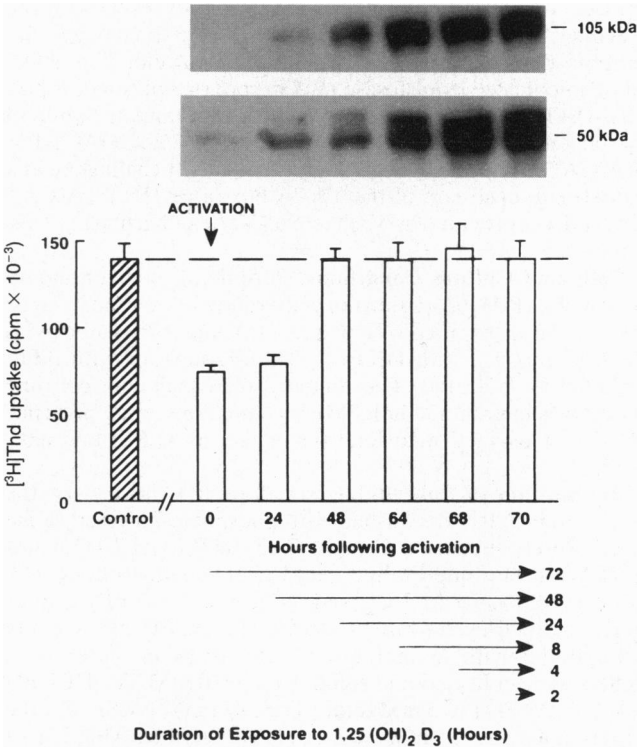

FIG. 3. Time course analysis of the effects of $1,25(OH)_2D_3$ on NF-κB and cell proliferation in PHA-activated human lymphocytes. PBMCs were activated for 72 hr with PHA, and 10^{-8} M $1,25(OH)_2D_3$ was added either simultaneously with addition of PHA or 24, 48, 64, 68, or 70 hr following addition of PHA. At the end of the 72-hr activation, cytosols were prepared by ultracentrifugation, and aliquots (100 μg of protein) were immunoblotted with the SP1157 Ab. Cell proliferation was assessed by [^{3}H]thymidine incorporation in triplicate aliquots (0.2×10^6 per 0.2 ml of medium) and was expressed as cpm $\times 10^{-3}$. The hatched bar indicates cell proliferation in PBMCs activated for 72 hr with PHA in the absence of $1,25(OH)_2D_3$.

Unstimulated lymphocytes used for the PHA activation experiment had no detectable c-rel; however, small levels of c-rel were present in the preparation used for the OKT3 activation experiment, perhaps reflecting an interindividual variation in the number of *in vivo* (or during the isolation procedure) activated cells, similar to the situation we had observed in the preparations used for the experiments shown in Figs. 1–3. In any event, activation by either PHA or OKT3 led to a large increase in the expression of c-rel, as well as the p50 and p105 proteins.

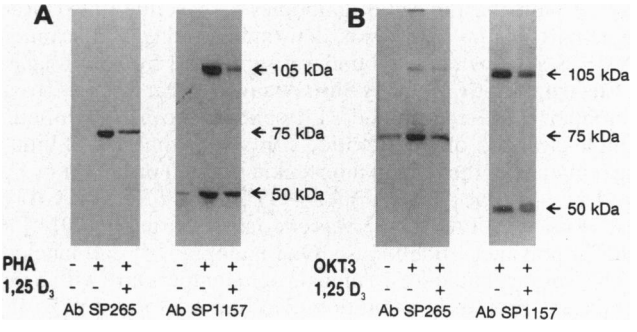

FIG. 4. Regulation of rel-related proteins in activated human lymphocytes by $1,25(OH)_2D_3$. PBMCs were incubated without stimuli or with PHA (*A*) or the OKT3 monoclonal Ab (*B*) in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$. At the end of 72 hr of incubation, cells were collected and cytosols were prepared by ultracentrifugation. Immunoblotting was performed with either the SP265 or SP1157 antibodies.

The inhibiting effect of $1,25(OH)_2D_3$ on NF-κB expression was confirmed with electrophoretic mobility shift assays using a synthetic oligonucleotide corresponding to the NF-κB response element (from the human IL-6 gene promoter) (Fig. 5). Nuclear extracts from resting cells did not contain proteins capable of retarding the consensus NF-κB DNA binding site (lane 2). However, nuclear extracts from cells activated with PHA formed a DNA–protein complex that appeared as a single retarded band (lane 3). $1,25(OH)_2D_3$ caused a significant reduction in the DNA–protein complex, as evidenced by the decrease in the intensity of this band (lane 4). This complex could be competitively inhibited by an excess of unlabeled NF-κB DNA binding motif (lanes 5 and 6) but not by an excess of a nonspecific oligonucleotide containing an AP-1 binding motif (lane 7).

Finally, to ascertain the functional significance of the inhibiting effect of $1,25(OH)_2D_3$ on NF-κB expression, we proceeded to examine whether $1,25(OH)_2D_3$ can affect the transcriptional activity of the NF-κB consensus sequence (Fig. 6). To do this, we transiently transfected the T-lymphocytic cell line Jurkat [which expresses the $1,25(OH)_2D_3$ receptor constitutively—i.e., without the requirement of activation (20)] with a construct containing the CAT gene under the regulatory control of the NF-κB consensus sequence that is present in the immunoglobulin κ light chain gene. Stimulation of the Jurkat cells for 8 hr with PHA resulted in an increase in the NF-κB-driven CAT expression. In the presence of $1,25(OH)_2D_3$, the increase in CAT activity was partially inhibited (39% ± 14%). Similar results (41% ± 5% inhibition as compared to untreated cells) were obtained in a separate experiment.

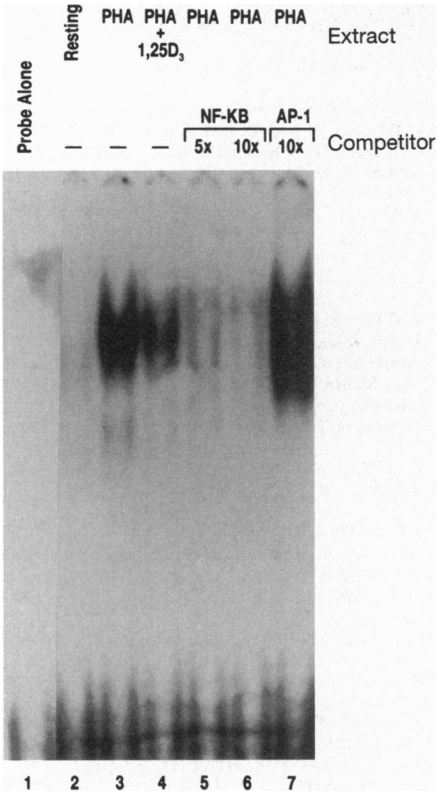

FIG. 5. $1,25(OH)_2D_3$ decreases NF-κB-DNA binding activity. PBMCs were incubated without or with PHA alone or in the presence of 10^{-8} M of $1,25(OH)_2D_3$ for 72 hr. At the end of incubation, nuclear extracts were prepared. Binding reactions were carried out for 30 min at room temperature using 5 μg of nuclear protein from resting cells (lane 2), PHA-activated cells (lanes 3 and 5–7), or cells activated with PHA in the presence of $1,25(OH)_2D_3$ (lane 4). In lanes 5–7, binding reactions were carried out in the presence of the indicated excess of unlabeled DNAs.

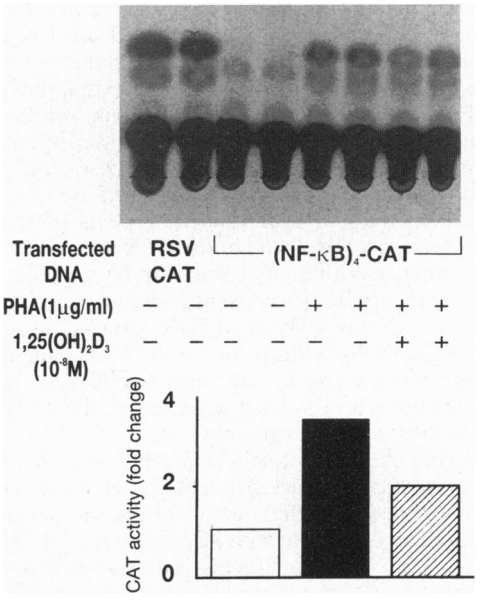

FIG. 6. $1,25(OH)_2D_3$ inhibits NF-κB regulatory sequence-driven transcription. Jurkat cells were transfected with the p(NF-κB)$_4$-CAT plasmid using the DEAE-dextran-mediated transfection method. After transfection cells were cultured in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$ for 48 hr and stimulated with PHA for the last 8 hr of the 48-hr period. Cell lysates were prepared, and CAT activity present in 100 μg of cellular protein was determined as percentage of acetylated [^{14}C]chloramphenicol. CAT activity of the control group was designated as 1×. Results shown are representative of two independent experiments. CAT activity in cells transfected with a plasmid containing the CAT gene driven by the Rous sarcoma virus (RSV-CAT) is also shown.

Treatment with $1,25(OH)_2D_3$ for 48 hr had no appreciable effect on [^{3}H]thymidine incorporation by unstimulated Jurkat cells but caused an ≈40% inhibition of [^{3}H]thymidine incorporation in cells stimulated with PHA.

DISCUSSION

These studies demonstrate that the abundance of p50, as well as its p105 precursor, and c-rel increase progressively during prolonged lymphocyte activation and that $1,25(OH)_2D_3$ downregulates these phenomena. Earlier studies had shown that the levels of the mRNA of genes encoding NF-κB subunits, including the p50 and c-rel, are enhanced in response to external stimuli such as antigens, cytokines, phorbol esters, and lectins (8, 21–24). In addition, observations by Hohmann *et al.* (25) had suggested that in lymphocytes and monocytic cells, preexisting NF-κB might be exhausted and active NF-κB can be obtained by *de novo* protein synthesis. The results of the Western blot analysis of this paper represent a direct demonstration of *de novo* synthesis of NF-κB during normal lymphocyte activation and are in agreement with these earlier observations.

Our data strongly suggest that the inhibiting effect of $1,25(OH)_2D_3$ on NF-κB is due to interference with new protein synthesis. This contention is supported by several pieces of evidence. (*i*) $1,25(OH)_2D_3$ decreased the levels of not only p50 but also its p105 precursor. (*ii*) Protein levels continued to show an increasing trend with time in the presence of $1,25(OH)_2D_3$, albeit at a lower level. (*iii*) $1,25(OH)_2D_3$ decreased the levels of p50 in the cytosolic and nuclear compartments—hence, excluding a mere effect of the hormone on the translocation of the protein between different cell compartments.

Consistent with the evidence that $1,25(OH)_2D_3$ inhibits NF-κB protein synthesis, we found that nuclear extracts from

human lymphocytes activated in the presence of $1,25(OH)_2D_3$ exhibited significantly less binding to an NF-κB DNA binding motif compared to lymphocytes activated in the absence of the hormone. Moreover, we have demonstrated that the inhibiting effect of $1,25(OH)_2D_3$ on NF-κB protein synthesis is of functional relevance to NF-κB-driven transcription. Indeed, activation of lymphocytes in the presence of $1,25(OH)_2D_3$ caused inhibition of the transcriptional activity of a minimal promoter consisting of four tandem repeats of the NF-κB consensus sequence. By virtue of the structure of this artificial promoter, its transcriptional activity can be regulated only by proteins that recognize this consensus sequence. It is therefore evident that $1,25(OH)_2D_3$ must have exerted its inhibiting effect on the activity of this promoter by decreasing the levels of such proteins induced following lymphocyte activation rather than through a direct interaction of the $1,25(OH)_2D_3$ receptor with this DNA segment.

The rank of potency of vitamin D metabolites with $1,25(OH)_2D_3$ being most effective (Fig. 2) and the promoter studies of Fig. 6 support the view that the vitamin D receptor mediates the effects seen here. Consistent with this, the effect of $1,25(OH)_2D_3$ required at least 4 hr. Nonetheless, studies involving specific antibodies to the vitamin D receptor will be required to prove the hypothesis that this protein mediates these effects.

In preliminary studies addressing the mechanism of action of $1,25(OH)_2D_3$ on NF-κB synthesis, we have found that $1,25(OH)_2D_3$ decreases the steady-state mRNA levels of the 4.4-kb transcript of p50 (26). However, using PBMCs from different subjects, we have found variations on the $1,25(OH)_2D_3$ effect at the mRNA abundance level. These and related observations suggest a very complicated mechanism of action that may involve actions at the transcriptional, translational, and posttranslational levels as well as at the level of the interaction between the p50 and its inhibitor, IκB, by affecting phosphorylation of the latter protein. The last possibility has been suggested by recent studies of Karin and colleagues (27) regarding the mechanism of glucocorticosteroid effects on NF-κB.

NF-κB enhances the expression of IL-2 and the IL-2 receptor, two molecules critical for lymphocyte proliferation (28). On the other hand, $1,25(OH)_2D_3$ inhibits IL-2 production and lymphocyte proliferation (1), raising the possibility that the antiproliferative effects of $1,25(OH)_2D_3$ could have been mediated via the inhibition of NF-κB. However, the time courses of the effects of $1,25(OH)_2D_3$ on NF-κB expression and lymphocyte proliferation were different. Specifically, whereas $1,25(OH)_2D_3$ could inhibit cell proliferation only when it was added within the first 24 hr following activation, $1,25(OH)_2D_3$ decreased p50 and p105 levels when added to the culture as late as 64 hr following addition of the activating agent. This suggests that the antiproliferative effect of $1,25(OH)_2D_3$ is not the result of its effects on NF-κB. This view is supported by the observation that $1,25(OH)_2D_3$ has no effect on the proliferation of lymphocytes activated by OKT3 (1), even though (as shown here) it inhibits NF-κB expression in OKT3-activated lymphocytes.

The relevance of the inhibiting effects of $1,25(OH)_2D_3$ on NF-κB proteins to the immunoregulating properties of this hormone is a matter of conjecture. Nevertheless, even though genes encoding NF-κB subunits are constitutively expressed in lymphocytes, new synthesis of these subunits would seem essential to replenish them, especially in situations where they might be rapidly consumed such as lymphocyte activation. Hence, induced expression of NF-κB subunits may be necessary to sustain nuclear NF-κB activity over longer periods of stimulation. In fact, based on evidence from studies on the transition from pre-B to B cells, it has been suggested that preexisting versus *de novo* synthesized NF-κB subunits play a causative role in activated lymphocyte function and in maintaining the activated state, respectively (4). The $1,25(OH)_2D_3$ receptor (and therefore responsiveness to this hormone) is not expressed until a few hours following lymphocyte activation (15). In view of these considerations, it is tempting to speculate that the expression of the $1,25(OH)_2D_3$ receptor following activation and the down-regulating effect of $1,25(OH)_2D_3$ on new synthesis of NF-κB-related proteins represent a biologic mechanism whereby excessive lymphocyte activation might be prevented.

In this report, we have obtained preliminary evidence that glucocorticosteroids, another class of steroid hormones with ubiquitous effects in biology including immunomodulating properties, exert effects similar to those of $1,25(OH)_2D_3$ on NF-κB expression. In line with this finding, Hass *et al.* (29) have shown that glucocorticosteroids inhibited phorbol 12-myristate 13-acetate-induced transient increase in NF-κB mRNA levels in the monocytic cell line U937 (29). Taken together, these observations raise the possibility that steroid hormones with widespread regulatory influences on a large variety of genes might be exerting part of their actions, indirectly, through their ability to modulate the expression of ubiquitous transcription factors, such as NF-κB.

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